PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 933-938 (1975)

Poly (Adnenosine Diphosphoribose) Synthase Activity of Isolated Nuclei of Normal and Leukemic Leukocytes (38930)

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Pyridine nucleotide levels are low in tumors and in tissues undergoing rapid cellular proliferation as compared to normal tissues (1, 2). The basis for the depressed level and its relation to cell replication is not clear. Although NAD^+ is a coenzyme for dehydrogenases, an alternate pathway of this nucleotide has been discovered. An enzymic system is present in rat liver nuclei or chromatin which transfers the ADPR[2] moiety of NAD^+ to nuclear proteins to form a biopolymer (3, 4). Evidence has been presented to show that the formation of this biopolymer might influence DNA synthesis (5, 6).

It was reported that poly(ADPR) synthase activity of isolated nuclei from Novikoff hepatoma was significantly higher than that of normal liver nuclei (7). When isolated liver nuclei were incubated with NAD^+ the template activity for DNA synthesis was inhibited; whereas the template of isolated nuclei from Novikoff hepatoma incubated with NAD^+ was not affected (7). In the present study poly(ADPR) synthase activities in isolated nuclei obtained from normal human leukocytes and leukemic leukocytes were determined. In addition isolated nuclei from normal and leukemic leukocytes were incubated with NAD^+ and their capacity to carry out DNA synthesis was determined

Materials and Methods. [Adenosine-^{3}H(G)]NAD^+ (15 Ci/mmole and [methyl-^{3}H]thymidine triphosphate (18.3 Cimmole) were purchased from New England Nuclear Corporation, Boston, Mass. [^{14}C-adenosine]NAD^+ (sp act 100 mCi/mmole) was purchased from Amersham/Searle Corp., Arlington Heights, Ill. [^{3}H]NAD^+ and [^{14}C]NAD^+ were used and the results were found to be identical. All deoxyribonucleotide triphosphates and NAD^+, Grade V, were purchased from Sigma Chemical Co., St. Louis, Mo. Venom phosphodiesterase from *Crotalus adamanteus*, pancreatic deoxyribonuclease, and spleen phosphodiesterase were purchased from Worthington Biochemical Corp., Freehold, NJ. [^{14}C]PR-AMP used as standard was a generous gift or Dr. T. Sugimura and Dr. M. Miwa, Tokyo, Japan. Normal white blood cells were purchased from the Blood Bank of New York, NY.

Preparation of leukocyte nuclei. Blood from leukemic patients was fractionated by passage through an IBM Experimental Blood Cell Separator, type 2990-6. The leukocyte-rich fraction was allowed to stand in the cold room overnight to sediment the cells. The leukocytes were pipetted off, washed with cold saline, and suspended in 10 vol of a medium of 0.4 m*M* sodium phosphate (pH 6.8), 2 m*M* $MgCl_2$, and left standing in the cold for 10 min. To the cell suspension 1 vol of a medium containing 0.64 *M* sucrose, 0.4 m*M* sodium phosphate (pH 6.8), 2 m*M* $MgCl_2$ was added. The cells were homogenized in a Dounce homogenizer using a tightly fitted pestle. After 20 strokes most of the cells were broken. The extent of the cytolysis was checked by microscopic examination of the cells stained with trypan blue. The homogenate was centrifuged at 1000*g* for 10 min and the sediment washed with a solution containing 0.32 *M* sucrose, 2 m*M* $MgCl_2$, 0.4 m*M* sodium phosphate (pH 6.8). The washed crude nuclear pellets were resuspended in a solution which contained 2.3 *M* sucrose, 2 m*M* $MgCl_2$, and the suspension centrifuged in a Spinco rotor SW 27

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[2] Abbreviations used: ADPR, adenosine diphosphoribose, PR-AMP, 2′-(5″-phosphoribosyl)-5′-AMP; TCA, trichloroacetic acid; ALL, acute lymphatic leukemia; CLL, chronic lymphocytic leukemia; AML, acute myelogenous leukemia.

for 60 min at 23,000 rpm. The pellet of purified nuclei was washed once and resuspended in a medium which contained 0.32 *M* sucrose, 2 m*M* $MgCl_2$. In some experiments, crude nuclei suspension obtained before the step of centrifugation in 2.3 *M* sucrose was used.

Assay for poly(ADPR) synthase activity. The assay system contained 80 m*M* Tris–HCl buffer (pH 7.5), 15 m*M* $MgCl_2$, nuclei preparation of 150–200 μg of protein, 2 m*M* [^{3}H]NAD^+ (5 μCi/mmole), or [^{14}C]NAD (1 μCi/mmole) in a total vol of 0.25 ml. The mixture was incubated at 25° for 20 min and processed as described in a previous report (5). The counting efficiency for tritium was 40% and for [^{14}C] was 70%. To determine the distribution of [^{3}H]NAD^+ incorporated into various nuclear protein fractions, the reaction system was incubated at 25° for 60 min. The nuclei were fractionated as described by Steele and Busch (8).

Preparation of poly(ADPR) and estimation of chain length. Poly(ADPR) was prepared from nuclei incubated with [^{3}H]NAD at 25° for 60 min and analyzed as described by Shima *et al.* (9). After preincubation of nuclei with [^{3}H]NAD, the reaction was stopped by the addition of a solution containing 7% TCA and 1% $Na_2P_2O_7$. The insoluble precipitate was washed with the same acid solution, and three or four times with 95% ethanol. The product was resuspended in 0.1 *N* NaOH. The alkali mixture was incubated for 60 min at 37°, neutralized with HCl, and incubated with snake venom phosphodiesterase (50 μg/ml) for 50 min at 37°. The hydrolyzed mixture was spotted on paper and developed by descending chromatography with two systems: Isobutyric acid–NH_4OH–water (66:1:33) by volume and 0.1 *M* sodium phosphate buffer (pH 6.8):ammonium sulfate:1-propanol (100:60:1, v:w:v). Standard solutions of AMP and PR-AMP were run with each system and the amount of radioactivity in each spot determined. The average length of polymers was calculated as described by Shima *et al.* (9).

Other assays. The procedure for the determination of DNA synthesis is described in previous reports (5, 6). Protein was determined according to Lowry *et al.* (10) and DNA by the diphenylamine reaction (11).

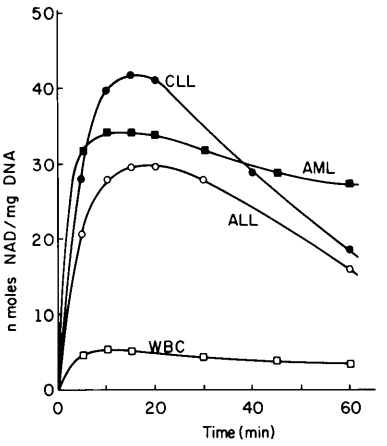

FIG. 1. Kinetics of [^{14}C]NAD or [^{3}H]NAD incorporation by different types of leukocytes nuclei. The procedure of incubation is described in Methods in the text. WBC, normal white blood cells; ALL, acute lymphocytic leukemia cells; AML, acute myelogenous leukemia cells; CLL, chronic lymphocytic leukemia. [^{14}C]NAD was incubated with CLL and WBC; [^{3}H]NAD with ALL and AML.

Results. Poly(ADPR) synthase activity was higher in leukemic leukocyte nuclei than in normal leukocyte nuclei (Fig. 1). In addition, the kinetics of incorporation of [^{3}H]ADPR from [^{3}H]NAD^+ by leukemic and by normal leukocyte nuclei were quite different (Fig. 1). With leukemic leukocyte nuclei the incorporation of [^{3}H]NAD^+ increased up to about 20 min of incubation and decreased thereafter, whereas the rate of incorporation was low with normal WBC nuclei and reached a plateau in 10 min (Fig. 1). The decline in the incorporation varied considerably with different specimens of leukemic leukocyte nuclei. The fall in the incorporation observed with leukemic leukocytes nuclei was probably complicated by an increase in the degradation of the polymers. Since poly(ADPR) is degraded by poly(ADPR) glycohydrolase and phosphodiesterase (4–6), the present results suggest that the activities of these two enzymes might be high in leukemic leukocyte nuclei. Preliminary results suggest that the degrading activity is due to a glycohydrolase.

To establish that polymers of ADPR

TABLE I. ENZYMATIC DIGESTION OF THE RADIOACTIVE MATERIAL INCORPORATED INTO NORMAL AND LEUKEMIC AND LEUKOCYTE NUCLEI INCUBATED WITH [^{14}C]NAD.

Enzymatic treatment	Radioactivity[a] (cts/min) Normal WBC	CLL cells
Control	387	2100
RNAse, pancreatic (100 μg/ml)	350	2074
DNAse, pancreatic (300 μg/ml)	372	1988
Venom phosphodiesterase (100 μg/ml)	7	42
Spleen phosphodiesterase (100 μg/ml)	320	1942

[a] Radioactivity in trichloroacetic acid-precipitable fraction.

were formed when isolated nuclei were incubated with [^{3}H]NAD^{+}, the radioactive nucleoproteins were treated with various hydrolytic enzymes (Table I). The radioactive materials incorporated into nucleoproteins were solubilized only on treatment with venom phosphodiesterase (Table I), suggesting that the incorporated radioactive material was poly(ADPR) since the polymers are hydrolyzed by venom phosphodiesterase and a specific poly(ADPR) glycohydrolase (4). To confirm the presence of oligomers or polymers of ADPR in the nucleoprotein fractions the radioactive materials were isolated and hydrolyzed with venom phosphodiesterase and the products analyzed by paper chromatography (9). The major products formed were identified as AMP and PR-AMP (Table II). The polymers synthesized by leukemic leukocyte nuclei were estimated to contain on the average about three to five residues per chain while those formed by normal leukocyte nuclei contained about two residues per chain.

The distribution of labeled ADPR in the various nucleoprotein fractions was determined by incubating isolated normal and leukemic leukocyte nuclei with [^{14}C]NAD^{+}. It was found that all of the nucleoprotein fractions contained radioactivity (Table III). The distribution of radioactivity in the nucleoprotein fractions obtained from normal leukocytes and CLL leukocytes nuclei was similar in that the nonhistone fractions of normal and leukemic nuclei contained the largest amount of labeled ADPR. This finding parallels the result obtained with Novikoff hepatoma nuclei (7) and differs from that observed with rat liver nuclei (5–7).

TABLE II. CHAIN LENGTH OF POLY(ADPR) SYNTHESIZED *In Vitro* BY ISOLATED NORMAL AND LEUKEMIC LEUKOCYTE NUCLEI[a].

Type of cell nuclei	PR-AMP (cts/min)	AMP (cts/min)	Chain length PR-AMP/AMP + 1
Normal	720	950	1.7
	405	248	2.6
Chronic lymphocytic Leukemia	3740	867	5.3
	2420	655	4.7
Acute myelogenous Leukemia	1108	523	3.1

[a] [^{3}H]NAD was used.

TABLE III. DISTRIBUTION OF RADIOACTIVITY IN VARIOUS FRACTIONS OF ISOLATED NUCLEI FROM NORMAL AND CHRONIC LYMPHOCYTIC LEUKEMIC LEUKOCYTES INCUBATED WITH [^{14}C]NAD^{+}.[a]

Nuclear protein fraction	Normal leukocytes 10^{-3} cts/min	mg protein	CLL leukocytes 10^{-3} cts/min	mg protein
Whole nuclei	186	7.7	1295	11.6
Nucleoplasmic proteins	8	1.8	54	3.4
Histones	24	2.6	137	3.0
Residual	138	2.9	942	3.3

[a] Isolated nuclei from normal and CLL leukocytes were incubated with [^{14}C]NAD as described in the text. At the end of the incubation the concentration of the NaCl in the medium was increased to 0.15 *M*. The mixture was centrifuged at 3000*g* for 10 min. The pellet was suspended in a medium containing 50 m*M* Tris-HCl (pH 8), 5 m*M* $MgCl_2$. The suspension was centrifuged at 3000*g* for 10 min. The extraction was repeated. The three supernatants were pooled and designated as the nucleoplasmic protein. The pellet was extracted with 0.25 *N* H_2SO_4 twice. The suspension was centrifuged at 3000*g* for 10 min. The supernatants were pooled and designated as the histone fraction. The pellet obtained after the histones were extracted was suspended in 20 m*M* sodium phosphate buffer (pH 6.0). This fraction was designated as the residual fraction.

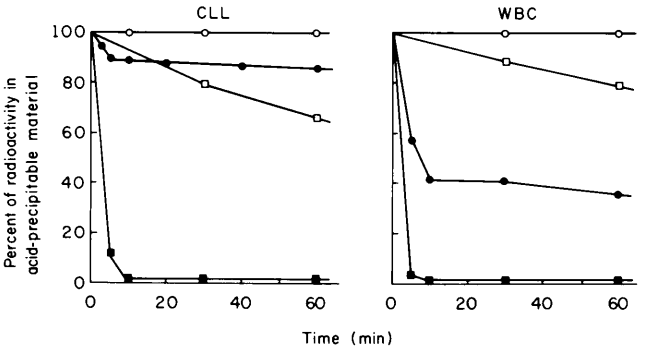

FIG. 2. Release of acid-precipitable radioactive material associated with histones and residual proteins on incubation with 0.1 *N* NaOH. CLL and WBC nuclei were incubated with [^{14}C]NAD. Histones and residual fractions were prepared as described under methods. Aliquots of histones (□) or residual proteins (○) were incubated in 50 m*M* sodium phosphate buffer (pH 7.0) and in 0.1 *N* NaOH (■, ●). The reaction was stopped by the addition of 10% trichloroacetic acid to the mixture containing residual proteins or by addition of 20% trichloroacetic acid to the mixture containing histones. The precipitated material was collected on a Millipore filter and the radioactivity measured in a Packard Scintillation Counter.

The initial ADPR moiety was shown to be bound to nucleoproteins by covalent linkage (3, 4). The bond was stable to acid treatment and labile under alkali condition (3, 4). The stability of the labeled ADPR incorporated in histones and residual fraction (see Table III) obtained from CLL and normal leukocyte nuclei was determined. These fractions were incubated in 25 m*M* phosphate buffer (pH 7.0) or in 0.1 NaOH at 37° for various periods of time. To each sample 20% TCA was added to precipitate the proteins (Fig. 2). A large part of the radioactivity incorporated in histones of normal and CLL leukocyte nuclei became acid-soluble after alkali treatment (Fig. 2). On the other hand, the major part of the radioactive material incorporated in the nonhistone proteins was TCA-precipitable after alkali treatment (Fig. 2). About 6 and 60% of the radioactive materials incorporated in CLL and WBC residual fractions, respectively, became acid-soluble after alkali treatment. These results suggest that poly(ADPR) might be linked to proteins by different types of bonds with varying degrees of lability to alkali treatment or that the released polymers were large enough to be precipitated by TCA. To determine which of these two proposed hypotheses were correct, the next set of experiments was conducted. The residual fraction of CLL nuclei (See Table III) was suspended in 0.1 *M* NaOH and incubated for 60 min at 37°. The mixture was neutralized with HCl, placed in ice for 15 min, and centrifuged at 25,000*g* for 20 min. The amount of DNA, protein, and radioactivity in the sediment was 84, 76, and less than 6% of the control values, respectively. Most of the radioactivity was located in the supernatant and about 50% of it remained insoluble after treatment with 20% TCA. The present results suggest that long chain polymers were dissociated from nonhistone proteins by alkali treatment.

Since incubation of isolated rat liver nuclei with NAD^+ affected a marked inhibition of the DNA synthetic activity (4, 5), normal leukocyte and leukemic leukocyte nuclei were incubated with NAD^+ and the ability to carry out DNA synthesis determined (Table IV). The endogenous DNA synthesis of nuclei incubated with and without NAD^+ were the same with both types of nuclei.

Discussion. The present finding that poly(ADPR) synthase activities of leukemic leukocyte nuclei were significantly greater than that of normal leukocyte nuclei is similar to the previous observation that hepatoma nuclei possessed greater enzymic activity than liver cell nuclei (7). The high poly(ADPR) synthase activity may be related to the low levels of NAD^+ in tumors and in growing tissues (1, 2). It has been reported that polymers of ADPR are formed

TABLE IV. DNA SYNTHETIC ACTIVITY OF ISOLATED NUCLEI INCUBATED WITH NAD^+

Type of nuclei	DNA synthesis [^{3}H]TMP (cpm)/ 100 μg DNA	
	Control	NAD treated
Normal leukocytes	570	610
	420	400
	730	710
Chronic lymphatic leukocytes	1980	1740
	2440	2680
	1125	980
	1107	2274
Acute myelogenous leukocytes	916	885
	1742	1605

during the cell cycle (12). This finding suggests that growing tissue may utilize NAD^+ to form poly(ADPR) which would tend to depress the tissue level of NAD^+. It should be pointed out, however, that the exact function of poly(ADPR) in the cell cycle has not been established.

An interesting finding was that the length of the polymers synthesized by nuclei of leukemic leukocytes was always longer than the chains synthesized by normal leukocytes nuclei (Table II). This finding was also confirmed by the experiment shown in Fig. 2. Most of the radioactivity associated with the residual fraction from CLL nuclei was TCA-insoluble after the polymers were released from the proteins with 0.1 *N* NaOH treatment. This finding needs to be reinvestigated.

When isolated nuclei of normal and leukemic leukocytes were incubated with NAD^+, the DNA synthetic activity was not affected (Table IV). This finding parallels the results obtained with Novikoff hepatoma nuclei (7), and is at variance with that of rat liver nuclei, whereby the DNA synthetic activity was inhibited (5, 7). Lehman and Shall (13) reported that the incorporation of TTP in a DNA polymerase assay system by isolated nuclei of mouse L5178Y lymphoma cells and normal pig lymphocytes stimulated or unstimulated with phytohemagglutinin was not significantly inhibited on preincubation with NAD^+. Furthermore, Roberts *et al.* (14) reported that ADP-ribosylation of nuclear protein of HeLa cells caused an enhancement of the template activity for DNA synthesis. These apparently diversified results might arise from the fact that different tissues were being studied and that the basis for the effect of poly(ADPR) on DNA synthesis might be a variable phenomenon. It has been demonstrated that poly(ADPR) formation affected a release of DNA polymerase from rat liver chromatin (15), inhibited rat liver and bull semen alkaline Ca^{2+}, Mg^{2+}-dependent endonuclease activity (6, 16), and stimulated rat testis acid endonuclease activity (17).

Summary. Poly(adenosine diphosphoribose) (ADPR) synthase activities of nuclei isolated from normal human and leukemic leukocytes were assayed by incubation with radioactive NAD^+. The synthase activity of leukemic leukocyte nuclei was significantly higher than that of normal leukocyte nuclei. The average length of polymers formed by isolated leukemic nuclei under the prescribed experimental condition ranged from 3.1 to 5.3 ADPR residues per chain, while those produced by normal leukocytes nuclei was 1.7 and 2.6 residues per chain.

Isolated leukemic and normal leukocyte nuclei were incubated with and without NAD^+ and the ability to carry out DNA synthesis was measured. The endogenous DNA synthesis of NAD-treated and untreated nuclei was the same. This finding parallels the result obtained with Novikoff hepatoma cell nuclei and differs from the observation with rat liver or testis nuclei.

The authors are grateful to Mrs. M. Elvira for the technical assistance. The study was supported in part by USPHS Grant P01 HD05671, from The National Institute of Child Health and Human Development.

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Received December 12, 1974. P.S.E.B.M., 1975, Vol. 149.